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(54) Title: NEW MUTATIONAL PROFILES IN HIV-1 REVERSE TRANSCRIPTASE CORRELATED WITH PHENOTYPIC DRUG RESISTANCE

(57) Abstract: The invention provides novel mutations, mutation combinations or mutational profiles of HIV-1 reverse transcriptase and/or protease genes correlated with phenotypic resistance to HIV drugs. More particularly, the present invention relates to the use of genotypic characterization of a target population of HIV and the subsequent correlation of this information to phenotypic interpretation in order to correlate virus mutational profiles with drug resistance. The invention also relates to methods of utilizing the mutational profiles of the invention in databases, drug development, i.e., drug design, and drug modification, therapy and treatment design, clinical management and diagnostic analysis.

## NEW MUTATIONAL PROFILES IN HIV-1 REVERSE TRANSCRIPTASE CORRELATED WITH PHENOTYPIC DRUG RESISTANCE

The present invention is directed to the field of nucleic acid diagnostics and the identification of base variation in target nucleic acid sequences. More particularly, the present invention relates to the use of such genotypic characterization of a target population of HIV and the subsequent association, *i.e.*, correlation, of this information to phenotypic interpretation in order to correlate virus mutational profiles with drug resistance. The invention also relates to methods of utilizing the mutational profiles of the invention in drug development, *i.e.*, drug design, drug modification, and drug development, therapy and treatment design, clinical management and diagnostic analysis.

Retroviral inhibitors may block viral replication in various ways. For example, Nucleoside Reverse Transcriptase Inhibitors (NRTIs), compete with the natural nucleoside triphosphates for incorporation into elongating viral DNA by reverse transcriptase. Chemical modifications that distinguish these compounds from natural nucleosides result in DNA chain termination events. NRTIs that are currently available include zidovudine (ZDV), didanosine (ddl), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir (ABC).

Nucleotide reverse transcriptase inhibitors (NtRTIs) have the same mode of action as NRTIs, but they differ in that they are already monophosphorylated and therefore they require fewer metabolic steps. Adefovir (bis-POM-PMEA) and bis-POC PMPA belong to this category of treatments.

Non-Nucleoside Reverse Transcriptase inhibitor (NNRTIs) are a group of structurally diverse compounds which inhibit HIV reverse transcriptase by noncompetitive binding to or close to the active site of the viral reverse transcriptase enzyme, thereby inhibiting its activity. Available compounds in this group include nevirapine (NVP), delavirdine (DLV) and efavirenz.

Protease Inhibitors (PIs) are peptidomimetic and bind to the active site of the viral protease enzyme, thereby inhibiting the cleavage of precursor polyproteins necessary to produce the structural and enzymatic components of infectious virions. PIs that are currently available include saquinavir (SQV), ritonavir (RTV), indinavir (IDV) nelfinavir (NFV), amprenavir (APV) and ABT-378 (lopinavir).

The options for antiretroviral therapy have improved considerably as new agents have become available. Current guidelines for antiretroviral therapy recommend a triple combination therapy regimen for initial treatment, such as one PI and 2 NRTIs or one NNRTI and 2 NRTIs. These combination regimens show potent antiretroviral activity and are referred to as HAART (highly active antiviral therapy).

Additionally, the development and standardization of plasma HIV-1 RNA quantification assays has led to the use of viral load measurements as a key therapy response monitoring tool. The goal of antiretroviral therapy is to reduce plasma viremia to below the limit of detection on a long-term basis. However, in a significant number of 5 patients, maximal suppression of virus replication is not achieved and for those in whom this goal is reached, a significant number experience viral load rebound. Viral load data provide no information on the cause of the failure.

Why therapies fail may be due to a number of factors, including insufficient 10 antiviral activity of the regimen, individual variations in drug metabolism and pharmacodynamics, difficulties in adhering to dosing regimen, requirements for treatment interruption due to toxicity, and viral drug resistance. Moreover, drug 15 resistance may develop in a patient treated with sub-optimal antiretroviral therapy or a patient may be infected with drug-resistant HIV-1. Although drug resistance may not be the primary reason for therapy failure, in many cases any situation which permits viral replication in the presence of an inhibitor sets the stage for selection of resistant variants.

Viral drug resistance can be defined as any change in the virus that improves 20 replication in the presence of an inhibitor. HIV-1 drug resistance was first described in 1989 and involved patients that had been treated with zidovudine monotherapy, which represented the only treatment option at that time. See Larder, B.A., et al., Science 243, 1731-1734 (1989). Emergence of resistance is almost always being observed during the course of treatment of patients with single antiretroviral drugs. Similarly, *in vitro* 25 passage of viral cultures through several rounds of replication in the presence of antiretroviral compounds leads to the selection of viruses whose replication cycle is no longer susceptible to the compounds used. Resistance development has also been observed with the introduction of dual NRTI combination therapy as well as during the administering of the more potent NNRTIs and PIs. Individual antiretroviral agents differ in the rate at which resistance develops: selection for resistant variants may occur within weeks of treatment or resistance may emerge after a longer treatment period.

Extensive genetic analysis of resistant viral isolates generated through *in vivo* or 30 *in vitro* selection has revealed that resistance is generally caused by mutations altering the nucleotide sequence at some specific site(s) of the viral genome. The mutational patterns that have been observed and reported for HIV-1 and that are correlated with drug resistance are very diverse: some antiretroviral agents require only one single 35 genetic change, while others require multiple mutations for resistance to appear. A summary of mutations in the HIV genome correlated with drug resistance has been compiled. See Schinazi, R.F., Larder, B.A. & Meliors, J.W. 1997. Int. Antiviral News, 5, 129-142 (1997). Additionally, an electronic listing with mutations has also become

available at <http://hiv-web.lanl.gov>, [www.hivb.stanford.edu](http://www.hivb.stanford.edu), and <http://www.hivresistanceweb.com>.

It should be noted that the degree of susceptibility of a genetic variant to an antiretroviral compound is expressed herein relative to the wild-type virus (HIV 5 III/B/LAI reference sequence) as found, for example, in GenBank, the sequence of which is hereby incorporated by reference. Susceptibilities are generally expressed as ratios of IC<sub>50</sub> or IC<sub>90</sub> values (the IC<sub>50</sub> or IC<sub>90</sub> value being the drug concentration at which 50% or 90% respectively of the viral population is inhibited from replicating). Additionally, the genetic mutation is normally written as in reference to the wild type 10 virus, *i.e.*, K101N refers to replacement of a Lysine at codon 101 with a Asparagine. However, the mutations of the invention do not depend on the wild-type example listed in order to be within the practice of the invention. For example, the mutation 101N, refers to an Asparagine at the 101 codon regardless of the whether there was a Lysine at 101 prior to mutation.

15 Of course, as antiretroviral drugs are administered for longer periods of time, mostly in combination with each other, and as new antiretrovirals are being developed and added to the present drugs, new resistance-correlated genetic variants are being discovered. Of particular import is that the combination of antiretroviral agents can influence resistance characteristics. For example, different NNRTI resistance-correlated 20 mutations were selected on NNRTI-zidovudine combination therapy and different NRTI resistance-correlated mutations were selected in dual NRTI combination therapy. In the latter case, the result is high-level multi-drug resistance to all NRTIs.

Moreover, once viral resistance has developed, salvage therapy options may be severely restricted due to cross-resistance within each drug class. Recently, interest has 25 been focused on the characterization of alterations in viral drug susceptibility for better clinical management. This is as important for initial treatment as for when a therapy change is called for in order to minimize the emergence of resistance and improve the long-term prognosis of the patient. The choice of therapy regimen will be supported by knowledge of the resistance profile of the circulating virus population. Additionally, 30 therapy combinations will have a greater chance of being effective if they include agents that have a demonstrated potential of suppressing a particular virus population.

To achieve these and other advantages, and in accordance with the purpose of the invention as embodied and broadly described herein, the present invention, in one aspect, provides a computer system comprising a database correlating the presence of 35 at least one mutation in an HIV reverse transcriptase and the resistance of at least one strain of HIV to a reverse transcriptase inhibitor (RTI); and/or a database correlating the presence of at least one mutation in an HIV protease and the resistance of at least one

strain of HIV to a protease inhibitor (PI). More specifically, the database comprises a set of records corresponding to a correlation between a mutation and drug resistance.

In a further embodiment, the invention provides for a method of identifying drugs effective against NNRTI or NRTI resistant strains of HIV, the method

- 5 comprising the steps of: providing at least one strain of HIV comprising HIV reverse transcriptase containing at least one mutation described herein, determining the phenotypic response of the drug to the strain of HIV, and using the phenotypic response to determine the effectiveness of the drug. In an even further embodiment, the invention provides a method of identifying drugs effective against protease inhibitor
- 10 (PI) resistant strains of HIV, wherein the strain of HIV comprises HIV protease containing at least one mutation described herein, determining the phenotypic response of said drug to said strain of HIV, and using the phenotypic response to determine the effectiveness of the drug. In another embodiment, the invention provides for the drug identified using the methods of the invention.
- 15 The invention also provides for a method of designing therapy for treating patients infected with HIV comprising: collecting a sample from an HIV-infected patient; determining whether the sample comprises at least one nucleic acid encoding HIV reverse transcriptase having at least one mutation described herein or HIV protease having at least one mutation described herein; and using the presence of the nucleic acid
- 20 to design a therapy for the patient.

The invention also includes isolated HIV reverse transcriptase complexes resistant to at least one NNRTI or at least one NRTI comprising at least one mutation described above and an isolated HIV protease complex resistant to a PI comprising at least one mutation described above.

- 25 It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- 30 **Figure 1: Nucleoside analogue susceptibility of MDR patient-derived recombinant HIV variants.** Recombinant viruses were produced from patient plasma samples as described in Example 2 and tested for susceptibility to (a) d4T, (b) ddC and (c) ddl. The mean fold increase in IC<sub>50</sub> values (Mean fold resistance) relative to wild-type controls are shown for groups of viruses with different genotypes, i.e., the codon 151- M multi-drug resistance cluster (n=27), viruses with 69D/N (n=195), or 75M (n=43) in a background of AZT and 3TC resistance mutations and codon 69 insertion mutants (n=45) in a background of AZT resistance mutations. Error bars indicate standard errors. Note that the total number (n=310) is higher than the 302 MDR

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samples described because a small minority were 69D/N and 75M double mutants and are represented in both groups.

5 **Figure 2: Therapy histories of three patients whose HIV-1 isolates developed codon 69 insertions.** Nucleoside analogue therapies (AZT, 3TC, ddC, ddl or d4T) are shown as horizontal bars, indicating the time period in which each patient (1, 2 or 3) received a particular treatment. The time point at which plasma samples were obtained for genotypic and phenotypic analysis are shown by the arrows together with the specific codon 69 insertion detected. Any other therapies besides nucleosides that these patients may have been receiving are not indicated on this figure.

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#### DETAILED DESCRIPTION OF THE INVENTION

The invention, in one aspect, provides novel mutations or mutational profiles of HIV-1 reverse transcriptase and/or protease genes correlated with phenotypic resistance to anti-HIV drugs. More particularly, the present invention also relates to 15 the use of genotypic characterization of a target population of HIV and the subsequent correlation of this information to phenotypic interpretation in order to correlate virus mutational profiles with drug resistance. The invention also relates to methods of utilizing the mutational profiles of the invention in databases, drug development, *i.e.*, drug design, and drug modification, therapy and treatment design, clinical management 20 and diagnostic analysis.

The present invention concerns a computer system comprising:

at least one database correlating the presence of at least one mutation in a human immunodeficiency virus (HIV) reverse transcriptase and resistance of at least one strain of HIV to a reverse transcriptase inhibitor, comprising:

25 at least one set of records corresponding to a correlation between at least one mutation chosen from 44D, 77L, 115F, 118I, 184V, 208Y, 210W, 211K, 214F, 215F, 215Y, 219E, 219N, and 219Q, and resistance to d4T,

a record corresponding to a correlation between mutation 184I and resistance towards lamivudine,

30 at least one set of records corresponding to a correlation between at least one mutation chosen from 115F and 184V and resistance towards abacavir.

a combination of 62V, 75T, 77L, 116Y and 151M and resistance towards all nucleoside analogues

35 at least one set of records corresponding to a correlation between at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, and 190Q and resistance towards nevirapine,

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- at least one set of records corresponding to a correlation between at least one mutation chosen from 101H, 101P, 103H, 103N, 103S, 103T, 106M, 181C, 181S, 190Q and 236L and resistance towards delavirdine,
- 5 at least one set of records corresponding to a correlation between at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L and resistance towards efavirenz,
- a combination of 184V and 41L and 215Y, wherein the 184V resistance mutation reverses the effect of 41L and 215Y mutations on zidovudine
- 10 a 236L mutation, which increases the sensitivity towards nevirapine
- 15 at least one database correlating the presence of at least one mutation in a human immunodeficiency virus (HIV) protease and resistance of at least one strain of HIV to a protease inhibitor, comprising:
- at least one set of records corresponding to a correlation between at least one mutation selected from 54L, 54M, 54V, 84A, 84C and 84L and resistance towards a protease inhibitors selected from amprenavir, saquinavir, nelfinavir, ritonavir, indinavir.

The present invention further concerns a method of evaluating the effectiveness of an antiviral therapy of an HIV-infected patient comprising:

- (a) collecting a sample from an HIV-infected patient;
- 20 (b) determining whether the sample comprises at least one nucleic acid encoding HIV having at least one mutation selected from:
- i) a first nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from  
44D, 77L, 115F, 118I, 184V, 208Y, 210W, 211K, 214F, 215F, 215Y, 219E,  
25 219N, and 219Q, and resistance to d4T,  
184I and resistance towards lamivudine,  
115F and 184V and resistance towards abacavir.  
62V, 75T, 77L, 116Y and 151M and resistance towards all nucleoside analogues  
101H, 101P, 103H, 103S, 103T, 106M, 181S, and 190Q and resistance towards  
30 nevirapine,  
101H, 101P, 103H, 103N, 103S, 103T, 106M, 181C, 181S, 190Q and 236L and resistance towards delavirdine,  
101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L and resistance towards efavirenz,  
35 184V and 41L and 215Y, wherein the 184V resistance mutation reverses the effect of 41L and 215Y mutations on zidovudine  
236L mutation, which increases the sensitivity towards nevirapine

ii) a second nucleic acid encoding a HIV protease having at least one mutation selected from: 54L, 54M, 54V, 84A, 84C and 84L and resistance towards a protease inhibitors selected from amprenavir, saquinavir, nelfinavir, ritonavir, indinavir.

(c) using the presence of said at least one nucleic acid to evaluate the 5 effectiveness of said antiviral therapy.

The present invention further concerns a method of identifying a drug effective against drug resistant strains of HIV, comprising:

i) providing at least one strain of HIV comprising:

10 a) at least one HIV reverse transcriptase containing at least one mutation chosen from:  
44D, 77L, 115F, 118I, 184V, 208Y, 210W, 211K, 214F, 215F, 215Y, 219E,  
219N, and 219Q, and resistance to d4T,  
184I and resistance towards lamivudine,  
115F and 184V and resistance towards abacavir.

15 62V, 75T, 77L, 116Y and 151M and resistance towards all nucleoside analogues  
101H, 101P, 103H, 103S, 103T, 106M, 181S, and 190Q and resistance towards  
nevirapine,

101H, 101P, 103H, 103N, 103S, 103T, 106M, 181C, 181S, 190Q and 236L and  
resistance towards delavirdine,

20 101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L and resistance  
towards efavirenz,

184V and 41L and 215Y, wherein the 184V resistance mutation reverses the  
effect of 41L and 215Y mutations on zidovudine  
236L mutation, which increases the sensitivity towards nevirapine

25 b) at least one strain of HIV comprising HIV protease containing at least one mutation  
chosen from: 54L, 54M, 54V, 84A, 84C and 84L and resistance towards a protease  
inhibitors selected from amprenavir, saquinavir, nelfinavir, ritonavir, indinavir.

ii) determining a phenotypic response of said drug to said strain of HIV; and  
iii) using said phenotypic response to determine the effectiveness of said drug.

30 The instant invention provides methods to identify drug effective for treating HIV  
infected individuals. The present invention further provides for phenotyping methods  
for assessing HIV therapy of an individual.

The instant invention further provides a method of designing a therapy for treating a  
35 patient infected with HIV comprising:

- i) collecting a sample from an HIV-infected patient;
- ii) determining whether the sample comprises

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- a) at least one nucleic acid encoding HIV reverse transcriptase having at least one mutation chosen from:  
44D, 77L, 115F, 118I, 184V, 208Y, 210W, 211K, 214F, 215F, 215Y, 219E,  
219N, and 219Q, and resistance to d4T,  
5 184I and resistance towards lamivudine,  
115F and 184V and resistance towards abacavir.  
62V, 75T, 77L, 116Y and 151M and resistance towards all nucleoside analogues  
101H, 101P, 103H, 103S, 103T, 106M, 181S, and 190Q and resistance towards  
nevirapine,  
10 101H, 101P, 103H, 103N, 103S, 103T, 106M, 181C, 181S, 190Q and 236L and  
resistance towards delavirdine,  
101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L and resistance  
towards efavirenz,  
184V and 41L and 215Y, wherein the 184V resistance mutation reverses the  
15 effect of 41L and 215Y mutations on zidovudine  
236L mutation, which increases the sensitivity towards nevirapine  
b) at least one nucleic acid encoding HIV protease having at least one  
mutation chosen from 54L, 54M, 54V, 84A, 84C and 84L and resistance towards a  
protease inhibitors selected from amprenavir, saquinavir, nelfinavir, ritonavir, indinavir.  
20 iii) using the presence of said at least one nucleic acid to design the therapy for  
said patient.
- Preferentially at least an additional mutation is present in the HIV protease nucleic acid  
selected from 10I, 20R, 20T, 24I, 33F, 33I, 33L, 36I, 46L, 71T, 71V, 77I, 77V, 82I,  
25 82V or 90M. More preferential, HIV protease nucleic acid having a mutation at codon  
54 comprises at least an additional mutation selected from codon 10 and 90 and confers  
resistance to a protease inhibitor selected from Amprenavir, Indinavir, Nelfinavir,  
Ritonavir and Saquinavir. In one embodiment the instant invention provides for a  
chimaeric HIV virus comprising at least one mutation of the instant invention  
30
- Not to be limited as to theory, the invention may utilize a combinatorial  
approach involving genotypic and phenotypic resistance testing to correlate mutations  
with resistance phenotypes. Without the specific combination of the technologies  
mentioned above, this correlation between mutation and resistance would not have been  
detected. In addition to the observation of these genotypic and phenotypic profiles in  
35 isolates from routine clinical practice, site-directed mutants were generated to confirm  
that these mutations actually form the basis of this pattern of drug resistance.

Resistance of HIV to antiretroviral drugs may be determined at the genotypic level by identifying mutations in the HIV-1 genome and by inferring the resistance of HIV-1 to antiretroviral drugs through searching for mutational patterns known to correlate with resistance. Alternatively, resistance of HIV to antiretroviral drugs may 5 be determined at the phenotypic level by culturing the virus in the presence of the inhibitors, and by measuring to what extent the drug inhibits viral replication. In this case, one measures the effect of all mutational interactions, the effects of genetic changes as yet unknown or not previously identified, the effect of the background genotype, etc., on the phenotype. Assays for detection of mutations in HIV-1 may be 10 based on polymerase chain reaction (PCR) amplification of viral genomic sequences. These amplified sequences are then analyzed using either hybridization or sequencing techniques. Hybridization-based assays include primer-specific PCR, which makes use of synthetic oligonucleotides designed to allow selective priming of DNA synthesis. 15 See Larder, B.A., et al., AIDS 5, 137-144 (1991); Richman, D.D., et al., J. Infect. Dis. 164, 1075-1081 (1991); Gingeras, T.R., et al., J. Infect. Dis. 164, 1066-1074 (1991). Only when primer sequences match the target sequence (wild-type or mutant) at the 3' end, is amplification of target sequences possible and DNA fragments are produced. Knowledge of the primer sequences allows one to infer the sequence of the viral isolate 20 under investigation, but only for the region covered by the primer sequences. Other hybridization-based assays include differential hybridization (Eastman, P.S., et al., J. Acq. Imm. Def. Syndr. Human Retrovirol. 9, 264-273 (1995); Holodniy, M., et al., J. Virol. 69, 3510-3516 (1995); Eastman, P.S., et al., J. Clin. Micro. 33, 2777-2780 (1995).); Line Probe Assay (LiPAJ HIV-11 RT, Innogenetics) (Stuyver, L., et al., Antimicrob. Agents Chemotherap. 41, 284-291 (1997).); and GENECHIP® technology 25 (Affymetrix) (D'Aquila, R.T. Clin. Diagnost. Virol. 3, 299-316 (1995); Fodor, S.P.A. et al., Nature 364, 555-556 (1993); Fodor, S.P.A. Nature 227, 393-395 (1997). DNA sequencing assays, on the other hand, provides information on all nucleotides of the sequenced region. Target sequences are amplified by PCR. Sequencing results may be reported as amino acid changes at positions in the protease gene and the reverse 30 transcriptase gene compared to the wild-type reference sequence. The changes included in the genotyping report may be limited to mutations at positions known to manifest drug resistance-associated polymorphisms. Polymorphisms at positions not associated with drug resistance are not required.

35 Phenotyping assays measure the ability of a replicating virus to grow in the presence of specific inhibitors compared to a wild-type sensitive reference virus. Consequently, these assays directly measure the degree of viral resistance or susceptibility to specific inhibitors. Applicable phenotyping assays include but are not limited to: the PBMC (peripheral blood mononuclear cells) p24 Antigen Assay, which

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- was the first standardized assay for determination of viral drug resistance in clinical HIV-1 isolates (Japour, A.J., et al., Antimicrob. Agents Chemother. 37, 1095-1101 (1993); Kusumi, K. et al., J. Virol. 66, 875-885 (1992); and the Recombinant Virus Assays (RVAs) which was first described as an alternative means of assessing 5 phenotypic resistance to RT-inhibitors (Kellam, P. & Larder, B.A., Antimicrob. Agents Chemother. 38, 23-30 (1994); and Pauwels, R., et al., 2nd International Workshop on HIV Drug Resistance and Treatment Strategies, Lake Maggiore, Italy. Abstr. 51(1998).

As is the case with the genotyping assays, the recombinant virus assay starts 10 with the amplification of viral target sequences by means of PCR. The amplicons are incorporated into a proviral laboratory clone with sequences homologous to those present in the amplicon deleted. This generates a stock of chimeric viruses. The viruses are tested for their ability to grow in the presence of different concentrations of drugs. Results are obtained by calculating IC<sub>50</sub> values for each inhibitor and by 15 reporting the results as IC<sub>50</sub> values, expressed in μM concentrations, or by computing the ratio of the IC<sub>50</sub> values found for the chimeric virus to the IC<sub>50</sub> values found for a wild type susceptible laboratory virus tested in parallel. In the latter case, resistance is expressed as "fold-resistance" compared to a wild-type susceptible HIV-1 strain. In order to meet the need for high-volume testing and a short turn-around time for an 20 individual test, the latest generation of phenotyping assays has undergone further modifications. The use of reporter gene systems for susceptibility testing allows the implementation of laboratory automation and standardization. See Pauwels, et al., J. Virol. Methods 20, 309-321 (1998); Paulous, S., et al., International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Florida, 25 USA. Abstr. 46 (1997); and Deeks, S. G., et al., 2nd International Workshop on HIV Drug Resistance and Treatment Strategies, Lake Maggiore, Italy. Abstr. 53 (1998).

The Antivirogram® assay (Virco) (WO 97/27480) is based on homologous recombination of patient derived HIV-1 gag/PR/RT sequences into a proviral HIV-1 clone correspondingly deleted for the gag/PR/RT sequences. See Pauwels, et al., J. Virol. Methods 20, 309-321 (1998). A similar assay (Phenosense ViroLogic) is based 30 on enzymatic ligation of patient-derived PR/RT sequences into a correspondingly deleted proviral vector carrying an indicator gene, luciferase, inserted in the deleted HIV-1 envelope gene. See Deeks, S. G., et al., 2nd International Workshop on HIV Drug Resistance and Treatment Strategies, Lake Maggiore, Italy. Abstr. 53 (1998). 35 Hertogs et al. Antimicrob. Agents Chemother. 44(3) 568-573 (2000) the disclosures of which are herein incorporated by reference.

To summarize, the development of high-throughput phenotyping and genotyping assays has allowed the establishment of a database containing the

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phenotypic resistance data and the genotypic sequences of over 30,000 clinical isolates.

Correlative data analysis and mutational cluster analysis of the database enables a search for mutational patterns with accompanying resistance. An example of which is virtual phenotyping (see PCT/EP01/04445).

- 5 In one embodiment, a neural network to accurately predict the development of therapeutic agent resistance or sensitivity based upon genotypic and phenotypic information and to accurately define the genetic basis of therapeutic agent resistance can be used. (see U.S. Patent Application No. 09/589,167 filed June 8, 2000, PCT/EP01/06360, the disclosure of which is expressly incorporated herein by reference 10 in its entirety).

Table 1 below lists some of the most commonly occurring resistance-correlated mutations appearing in clinical isolates after treatment with antiretroviral drugs.

- 15 Table 1: Examples of commonly occurring resistance-correlated mutations appearing in clinical isolates after treatment with antiretroviral drugs.

Protease Inhibitors		
Primary Mutations:	D30N M46L, V82A G48V, L90M V82A I50V	Nelfinavir Indinavir Saquinavir Ritonavir Amprenavir
Secondary Mutations:	L10I/F/R/V, K20R/M, L24I, V32I, L33F, M36I, M46I, I47V, I54V/L, L63P, A71V/T, G73S, V77I, V82A/F/T/S, I84V, N88D, L90M	
Compensatory Mutations	In a PI-resistant mutational background, mutations at <i>gag</i> cleavage site(s) may partially restore viral replicative Efficiency	
Reverse Transcriptase Inhibitors		
NRTI Mutations:	M41L, K65R, D67N, T69D, K70R, L74V, V75T/M, M184V, L210W, T215Y/F, K219Q/E	
MDR Mutations:	A62V, V75I, F77L, F116Y, Q151M T69S with associated insertions of 1 to 3 amino acids between codons 68 and 70 of RT	
NNRTI Mutations:	A98G, L100I, K101E, K103N/T, V106A, V108I, V179D/E, Y181C/I, Y188C/L/H, G190A, P225H, P236L	

Reversal Mutations:	<p>M184I/V decreases the effect of zidovudine resistance mutations M41 L and T215Y.</p> <p>L74V decreases the effect of zidovudine resistance mutation T215Y.</p> <p>K65R in mutational background (D67N, K70R, T215Y and K219Q) decreases zidovudine resistance.</p> <p>Y181C decreases the effect of zidovudine resistance mutation T215Y.</p>
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The invention contemplates resistance-correlated mutations to any type of HIV treatment therapy including but not limited to mutations conferring resistance to Protease Inhibitors and Reverse Transcriptase Inhibitors (NRTIs, NtRTIs, and

- 5 NNRTIs) in addition to Multi-Drug Resistant Mutations.

In one embodiment, the invention contemplates mutations conferring resistance to Protease Inhibitors (PIs). Table 1 lists two categories of mutations for all PIs: primary and secondary mutations. Primary mutations may be the major contributor to the development of resistance to a particular drug. Secondary mutations appear either

- 10 later during the course of therapy and also lead to resistance, or are already present as natural polymorphisms in a PI-naive viral isolate. A great number of secondary mutations enhance resistance to several PI-inhibitors simultaneously. This may lead to broad cross-resistance to this class of inhibitors, although subtle different phenotypic effects of those secondary mutations may exist.

- 15 Not to be limited as to theory, mutations occurring in the protease gene may impair cleaving efficiency of the polyprotein by the protease. Compensatory mutations have been found at the *gag* cleavage sites that allow more efficient cleaving of the sites by proteases that have mutated. Several studies of clinical isolates from protease-treated patients who have acquired PI resistance-correlated mutations have 20 shown mutations at *gag* p7/p1 and/or p1/p6 sites significantly raised the replicative efficiency of the mutant viruses.

- 25 Other mutations within the practice of the invention may confer resistance to NRTIs and NNRTIs. For example, the mutations typically conferring resistance to the NRTI zidovudine are M41L, D67N, K70R, L210W, T215Y and K219Q. Multiple mutations in HIV-1 reverse transcriptase also may confer high-level resistance to zidovudine and other NRTIs. Multiple mutations, when present, may act synergistically, and susceptibility decreases as the number of resistance-correlated mutations increases. For example, mutations correlated with resistance to didanosine are L74V, K65R. Resistance to lamivudine is also correlated with the emergence of 30 mutations M184V and M184I that confer very high resistance levels in addition to low-

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level resistance to didanosine, and zalcitabine. A low-level resistance to lamivudine may also be present in the absence of the 184 mutation while resistance to abacavir is correlated with mutations K65R, L74V, Y115F and M184V.

Another embodiment of the invention relates to multi-drug resistance mutations (MDR) and particularly MDRs to NRTIs. For example, the RT mutational constellation A62V, V75T, F77L, F116Y and Q151M together causes resistance to all nucleoside analogues.

Mutations conferring resistance to Non-Nucleoside Reverse Transcriptase inhibitor (NNRTIs) are also contemplated by the invention. For example, resistance-correlated mutations for nevirapine are A98G, L100I, K103N, V106A, V108I, Y181C/I, Y188C and G190A. These mutations are K103/N/T, Y181C and P236L for delavirdine and for resistance to efavirenz, the mutations are L100I, K101E, K103N, V108I, V179D, Y181C and Y188L.

Another aspect of the invention concerns reversal mutations. For example, the M184V lamivudine resistance mutation decreases the effect of zidovudine resistance mutations M41L and T215Y, while the L74V didanosine resistance mutation decreases the effect of zidovudine resistance mutation T215Y. Whether the described reversal effects are phenotypically significant or not, however, may depend on the combinations of mutations that are present.

In another embodiment, mutations may increase sensitivity to inhibitors. For example, the delavirdine mutation, P236L increases sensitivity of this mutant to inhibition by nevirapine and the lamivudine-resistance mutation M184V causes increased susceptibility to adefovir and to PMPA above the non-mutant sequence. This increased sensitivity seems to be reflected in an enhanced treatment outcome.

Novel mutations of HIV-1 reverse transcriptase (Table 2) within the practice of the invention, and their correlated phenotypic drug resistance, include but are not limited to those shown in Table 2.

Table 2: Novel RT Mutations and the Correlated Drug Resistance

Reverse Transcriptase Mutation	Resistant to:
41L	d4T
44D	d4T
62V	d4T
67N	d4T
69D	d4T
69N	d4T

Reverse Transcriptase Mutation	Resistant to:
69SXX	d4T
70R	d4T
75A	d4T
75I	d4T
75M	d4T
75T	d4T
77L	d4T
115F	d4T
116Y	d4T
118I	d4T
151M	d4T
184V	d4T
208Y	d4T
210W	d4T
211K	d4T
214F	d4T
215F	d4T
219E	d4T
219N	d4T
219Q	d4T
215Y	d4T

The existence of a single mutation or any combination of the mutations in Table 2 may confer resistance to d4T or one or more other treatments from the correlated class. Furthermore, if tools are used, such as those described herein, one may take the 5 identified mutation and the correlated-resistance to a class of treatment. Therefore, the invention also provides that the listed mutations and new combination of mutations, armed with the correlated class of drug, can be used to predict new resistance phenotypes such as resistance to additional PIs, NRTIs, NNRTIs, or MDR resistance. Additionally, the existence of a combination of mutations may confer the same or a 10 different drug resistance profile.

The present invention is also drawn to methods of using the correlations of the invention. In one embodiment, the invention provides for a database comprising the

correlation between: the presence of at least one mutation in HIV reverse transcriptase and the resistance of at least one strain of HIV to a reverse transcriptase inhibitor (RTI); or the presence of at least one mutation in HIV protease and the resistance of at least one strain of HIV to a protease inhibitor (PI).

5 In a further embodiment, the database may assist a physician in developing a treatment program or in determining the appropriate HIV therapy or combination therapy. For example, the VirtualPhenotype® assay system is a diagnostic tool for monitoring HIV-1 drug resistance. The system can be used for studying resistance development in clinical trials of anti-HIV-1 drugs, for improved clinical management of  
10 HIV-1 infected patients and for studying epidemiological aspects of drug resistance. It allows for a rapid determination of the drug sensitivity of the HIV-1 population circulating in the plasma of patients who have been exposed to antiretroviral drugs or who have been infected with drug resistant HIV-1 strains.

15 The invention also provides for a method of monitoring HIV-1 drug resistance using a method such as the one used in the VirtualPhenotype®, which combines in one test the determination of the genetic sequence of patient-derived HIV-1 genetic material and the interpretation of sequence variations found in the patient HIV strain with respect to the possible existence of antiviral drug resistance. In one embodiment, mutations associated with resistance to the different nucleoside reverse transcriptase  
20 inhibitors zidovudine, (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir, the nucleotide reverse transcriptase inhibitor adefovir (PMEA), the non-nucleoside reverse transcriptase inhibitors nevirapine, delavirdine and efavirenz, and the protease inhibitors saquinavir, ritonavir, indinavir and nelfinavir, are evaluated.

25 The methods of monitoring HIV-1 drug resistance, may also be used in combination with phenotypic drug resistance testing of viral isolates. For example, in one embodiment, a phenotypic test is utilized that is based upon the construction of chimeric HIV-1 strains composed of the protease (PR) and reverse transcriptase (RT) gene sequences which are isolated and amplified from the patient viral RNA. These  
30 strains may subsequently be recombined inside CD4+ T cells with a standard laboratory isogenic (HXB2) HIV-1 DNA construct from which the PR/RT gene sequences were deleted. The recombinant strains may then be grown in the presence of the above-mentioned antiviral drugs and the susceptibility of the viral isolates may be expressed as fold-change value of the IC50 of the drug on the patient isolates over the  
35 IC50 of the drug on a wild-type laboratory reference strain.

In one embodiment, the sample to be tested is prepared from a patient and the genotypic assay is performed through automated population-based full-sequence analysis (ABI). Therefore, the sequencing method used may provide information on all

nucleotides of the sequenced region. Sequencing results may be reported as amino acid changes at positions in the protease gene and the reverse transcriptase gene compared to the wild-type reference sequence. The changes included in the genotyping report may be limited to mutations at positions known to manifest drug resistance-associated 5 polymorphisms. Polymorphisms at positions not associated with drug resistance are not required.

In an even further embodiment, a report may be generated that shows the region of the patient virus that has been sequenced, the mutations detected by the test, and/or an interpretation of the evidence obtained. The interpretation may include the 10. antiretroviral drugs, the drug(s) for which a known resistance-associated mutation has been identified and/or to what extent the observed mutations are indicative of resistance to the drugs.

Knowledge of correlated geno- and phenotypes, together with knowledge of the catalytic site on the viral target for new compounds may also be utilized to tailor the 15 construction of new molecules and the implementation of new (combination) treatments for HIV.

In another embodiment, the invention is drawn to a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: collecting a sample from an HIV-infected patient; and determining whether the sample comprises 20 at least one nucleic acid encoding HIV reverse transcriptase having at least one mutation or HIV protease having at least one mutation. The sample may be a plasma sample, blood cells, or other tissue. Further, the invention has the potential to ameliorate HIV genotypic resistance diagnostics and can, in principle, lead to a better therapy and, under certain conditions, even be life saving.

25 In a further embodiment, the invention provides for a method of identifying or designing drugs effective against NNRTI or NRTI resistant HIV, the method comprising the steps of: providing at least one strain of HIV comprising a nucleic acid encoding HIV reverse transcriptase containing at least one mutation, and determining the phenotypic response of the HIV strain to a drug. In an even further embodiment, 30 the invention provides a method of identifying drugs effective against PI resistant strains of HIV, wherein the strain of HIV comprises HIV protease containing at least one mutation, and determining the phenotypic response of said strain of HIV to said drug. The invention is also useful for interpretation of resistance of HIV isolates. It can also be used in full sequence analysis of HIV. In addition, the invention has 35 applications for hybridization-based HIV analyses or in drug design, development, testing and marketing. In a further embodiment, the invention includes the drugs designed by the methods of the invention.

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The invention also provides for a method of designing therapy for treating patients infected with HIV comprising correlating the presence of HIV reverse transcriptase having at least one mutation described above with resistance to at least one NNRTI or at least one NRTI, or correlating the presence of HIV protease having at least one mutation with resistance to at least one PI.

The identification of the comparative mutations of the invention may lead to improved antiretroviral drug treatment programs. As outlined above, there is ample evidence demonstrating that poor virologic response to drug therapy may be correlated with the existence of genotypic and/or phenotypic viral resistance to one, several, or in 10 the worst case, all available antiretroviral drugs. As a consequence, resistance testing using the correlations of the invention may be used as a tool for identifying those drugs that no longer contribute towards decreasing the plasma viral load.

#### Examples

15 Example 1. The Identification of Mutational Patterns in HIV-1 Reverse Transcriptase and the Correlated Phenotypic Resistance.

Plasma samples were obtained from HIV-1-infected individuals from routine clinical practice in Europe and the US and were shipped to the laboratory on dry ice and stored at -70°C until analysis. Phenotypic analysis was performed using the 20 recombinant virus assay. See Kellam, P., and B. A. Larder. Antimicrob Agents Chemother 38:23-30 (1994); Hertogs, K., et al., Agents Chemother, 42:269-276 (1998); Briefly, protease (PR) and reverse transcriptase (RT) coding sequences were amplified from patient-derived viral RNA with HIV-1 specific primers. After homologous recombination of amplicons into a PR-RT deleted proviral clone, the resulting 25 recombinant viruses were harvested, titrated and used for *in vitro* susceptibility testing to antiretroviral drugs. The results of this analysis were expressed as fold-resistance values, reflecting the fold-increase in mean IC<sub>50</sub> (μM) of a particular drug when tested with patient-derived recombinant virus isolates, relative to the mean IC<sub>50</sub> (μM) of the same drug obtained when tested with a reference wild-type virus isolate (IIIB/LAI).

30 Genotypic analysis was performed by automated population-based full-sequence analysis (ABI). Results of the genotypic analysis are reported as amino acid changes at positions along the reverse transcriptase gene compared to the wild-type (HXB2) reference sequence. Cluster analysis by VirtualPhenotype® interpretational allowed detection of the occurrence of mutational pattern in the database containing the 35 genetic sequences of the clinical isolates and linkage with the corresponding resistance profiles of the same isolates. (See PCT EP01/04445)

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For the modeling studies, mutations were generated in the RT gene of HXB2, a wild-type laboratory HIV-1 strain, using the QuikChange<sup>1</sup> Site-Directed Mutagenesis Kit, STRATAGENE<sup>7</sup>, Stratagene Cloning systems, La Jolla, California, USA.

5     **Analysis of the clinical isolates**

Table 3 reports the frequency of mutations 44D/A, 118I, 184V, 215Y, and 41L in RT in clinical isolates with various levels of phenotypic resistance to zidovudine (ZDV) and lamivudine (3TC). The mutant isolates described here were drawn from a pool of clinical isolates.

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Table 3 reports the frequency of mutations 44D/A, 118I, 184V, 215Y, and 41L in RT in clinical isolates with various levels of phenotypic resistance to zidovudine (ZDV) and lamivudine (3TC). The mutant isolates described here were drawn from a pool of clinical isolates.

15

Table 3. Frequency of ZDV and 3TC Resistance-correlated Mutations in Clinical Isolates Susceptible or Resistant to ZDV and/or 3TC Compared to a Sample of Fully Susceptible Isolates

Resistance Class <sup>a</sup>	Frequency (%) of mutations					
	ZDV resistance-correlated mutations		3TC resistance-correlated mutations			No. of sample s
	41L	215Y	184V	44D/A	118I	
ZDV (<4), 3TC (<4)	4.5	4.8	0	1.3	3.1	314
ZDV (<4), 3TC (>10)	18.3	18.8	90	1.3	6.3	240
ZDV (>10), 3TC (<4)	59.5	68.9	0	14.9	18.9	74
ZDV (>10), 3TC (<4, <50)	77	72.2	4	30.2	39.7	126
ZDV (>10), 3TC (>50)	77.5	66.9	84.1	28.5	37.8	151

20

<sup>a</sup>Resistance (in parentheses) is expressed as the fold increase in the mean IC<sub>50</sub> of the drug relative to the mean IC<sub>50</sub> of the same drug for a wild-type reference laboratory HIV-1 strain.

25

*Isolates that are susceptible (WT) to both ZDV and 3TC (n=195): the frequency of any of the six mutations listed above was low.*

*Isolates that are resistant to ZDV (>10-fold, n=220):* Table 3 shows that the ZDV resistance-correlated mutations 215Y, 41L and 70R were high in frequency in this category and throughout all 3TC resistance categories. Mutation 184V was the predominant mutation in the high-resistance 3TC class (> 50-fold), whereas 184V was 5 rare in the intermediate 3TC resistance group and absent in the low-level resistance group and the 3TC susceptible group. The mutations 44D/A and 118I were present in all 3TC resistance categories.

*Isolates that are resistant to 3TC (> 10-fold, n=295):* Table 3 shows that the frequency of the high-level 3TC resistance-correlated mutation, 184V, was high in all ZDV 10 resistance categories (low, intermediate and high) and was the predominant mutation in the ZDV susceptible and intermediate-resistance group. As the resistance to ZDV increased, so did the frequency of the ZDV resistance-correlated mutations 41L, 70R and 215Y, while the frequency of mutation 184V decreased. Mutations 44D/A and 118I also substantially increased in frequency as resistance to ZDV increased.

15 The results thus far show that low and intermediate resistance to 3TC was not related to the presence of mutation 184V. Indeed, this mutation was practically absent in these classes. Table 3 further indicates that mutations 44D/A and 118I were present in high frequencies only in the presence of ZDV resistance mutations 215Y, 41L and 70R. In the isolates that were susceptible to ZDV, the frequency of ZDV resistance-20 correlated mutations was low and 44A/D and 118I were also rare, even though 3TC resistance was greater than 10-fold. In this group the high frequency of 184V accounted for the resistance to 3TC.

#### Analysis of the mutants generated by site-directed mutagenesis

25 Table 4 shows the codon changes introduced into a wild-type HXB2 background together with the fold-resistance values obtained when the different mutants were tested in the drug susceptibility assay. All six mutants carrying mutation 184V were highly resistant to 3TC. Two of them carried both 44D/A and 118I, while all but one (SDM23) carried ZDV resistance-correlated mutations.

30

Table 4 3TC and ZDV resistance-correlated mutations and phenotypic resistance in mutants with site-directed mutations

Mutant	3TC resistance profile			AZT resistance profile		
	Mutation(s)	Fold resistance <sup>b</sup>	n <sup>b</sup>	Mutations	Fold resistance	
SDM05		4 (0.3) <sup>c</sup>		41L, 210W, 211K, 214F, 215Y	64(15.0)	5
SDM18		2 (0.6)		41L, 67N, 210W, 211K, 214F, 215Y	45(13.4)	3

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Mutant	3TC resistance profile			AZT resistance profile		
	Mutation(s)	Fold resistance <sup>a</sup>	n <sup>b</sup>	Mutations	Fold resistance	
SDM19		4(0.7)		41L, 67N, 69D, 210W, 211K, 214F, 215Y	46(18.2)	2
SDM28	44D	1 (0.1)			2(0.3)	6
SDM31	44D	22 (2.5)		41L, 67N, 210W, 211K, 214F, 215Y	48(11.8)	4
SDM32	44D	8 (2.2)		41L, 67N, 69D, 210W, 211K, 214F, 215Y	49 (5.9)	6
SDM29	118I	2 (0.2)			2 (0.4)	6
SDM33	118I	7 (1.0)		41L, 67N, 210W, 211K, 214F, 215Y	49 (8.0)	6
SDM34	118I	32 (3.9)		41L, 67N, 69D, 210W, 211K, 214F, 215Y	34 (14.4)	5
SDM30	44D, 118I	3 (0.3)			1 (0.4)	6
SDM35	44D, 118I	14 (1.4)		41L, 67N, 210W, 211K, 214F, 215Y	49 (9.6)	5
SDM36	44D, 118I	15 (2.1)		41L, 67N, 69D, 210W, 211K, 214D, 215Y	49 (10.6)	5
SDM22	184V	78 (16.3)		41L, 210W, 211K, 214F, 215Y	7 (0.9)	5
SDM23	184V	82 (13.6)			2 (0.5)	6
SDM24	184V	85 (14.2)		69S-S-S, 210W, 211K, 214D, 215Y	27 (16.2)	5
SDM26	184V	72 (13.8)		41L, 67N, 210W, 211K, 214F, 215Y	25 (1.4)	5
SDM38	184V, 44D, 118I	82 (13.5)		41L, 67N, 210W, 211K, 214F, 215Y	20 (4.1)	6
SDM39	184V, 44D, 118I	84 (13.9)		41L, 67N, 69D, 210W, 211K, 214F, 215Y	21 (5.3)	5

<sup>a</sup>Fold increase in the mean IC<sub>50</sub> of the drug relative to the mean IC<sub>50</sub> of the same drug for a wild-type reference laboratory HIV-1 strain.

<sup>b</sup>n, number of replicate tests run for each phenotype drug resistance determination.

<sup>c</sup>Standard errors are indicated in parentheses.

All of the mutants followed the predicted ZDV resistance or susceptibility pattern. At the same time, three mutants were generated with a change at codon 44, three with a change at codon 118 and three with a change at both codons 44 and 118. Within each of these three groups two mutants also carried changes at positions correlated with resistance to ZDV, whereas one mutant remained wild-type at those codons. The drug resistance values listed in Table 4 clearly show that the presence of mutations at codon 44 and 118, singly or together, can cause intermediate resistance to 3TC (8 to 32-fold), distinguishable from the high resistance to 3TC (>62-fold) caused by mutation 184V. Moreover, the intermediate resistance to 3TC was only observed when mutations at positions 44 and/or 118 occurred in a ZDV-resistant background (41L, 67N, 210W,

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215Y) while resistance caused by mutation 184V was obviously not related to ZDV resistance.

**Relationship between the presence of changes at RT positions 44 or 118 in clinical samples and antiretroviral therapy**

5 As can be deduced from Table 4, changes at position 44 and 118 may occur in virus samples with or without the M184V substitution, but they appeared at higher incidence in samples with ZDV resistance. It was therefore of interest to look at the antiretroviral treatments administered to patients with HIV-isolates that contained 44D or 118I. We identified a subset of 86 samples with 44D and 88 samples with 118I  
10 originating from patients for whom antiretroviral histories were available. Although it was not possible to draw conclusions regarding the incidence of changes at 44 or 118 from this subset according to treatment history, as this was not a randomized study, this analysis nevertheless shed some light on the conditions that may lead to mutations at these positions.

15 For the 44D subset, 50/86 of the samples originated from patients who were receiving lamivudine at the sample date and 5 patients in this subset had never received 3TC prior and up to the sample date. All 5 patients had received zidovudine/didanosine at some time and all HIV-isolates were wild-type at position 184. The zidovudine treatment experience was extensive, as expected for historical  
20 reasons. All except one patient had received zidovudine in combination with other NRTI's and 70/86 had also received zidovudine monotherapy in the past. The one patient reported to be zidovudine naive had received stavudine. This sample contained 41L and 215Y.

25 Results for 118I subset were similar in that 55/88 samples originated from patients who were on lamivudine at the sample date and 2 patients had never received lamivudine (both had received zidovudine plus didanosine). Most patients, 83/88, had received zidovudine in combination with other NRTI's, and 70 had also received zidovudine monotherapy. The 5/88 zidovudine naive patients had received stavudine. For a few patients, consecutive samples showing the evolution of 44D or 118I were  
30 available.

These results indicate that mutations E44D/A and V118I in HIV-1 RT confer a low to intermediate level of resistance to 3TC when they occur in clinical isolates possessing a ZDV-resistant background. The cluster analysis of genotypically and phenotypically characterized clinical isolates and the results from the site-directed  
35 mutagenesis experiment confirm that indeed mutations at codons 44 and 118 are correlated with low and intermediate level of resistance to 3TC, with the restriction that ZDV resistance-correlated mutations be present. Additionally, the analysis of the clinical samples for which therapy histories were available and in which prior ZDV

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exposure was shown to be extensive, confirmed the results obtained from our large clinical data set in that mutations 44D/A and 118I appeared in the context of ZDV mutations.

5 Mutations 44D/A and 118I each are capable of independently generating resistance to 3TC. The experiment with site-directed mutagenesis does not indicate the existence of synergistic effects between the two mutants with respect to their phenotypic effect on 3TC resistance.

10 **Example 2. Determining the Genetic Basis of HIV-1 Multi-Nucleoside Resistance**

15 892 HIV-1 samples were surveyed in our resistance database from patients failing therapy using a standardized recombinant-based phenotypic assay and by DNA sequence analysis. Multi-nucleoside resistance was correlated with complex mutational patterns in the RT coding region. Plasma samples were obtained from patients who had received antiretroviral therapy. Selection was on the basis of a viral load >1000 HIV-1 RNA copies/ml and for the purpose of this study, patients with this level of plasma HIV-1 were considered to be failing therapy.

20 Viral RNA was extracted from 200  $\mu$ L patient plasma using the QIAAMP<sup>®</sup> Viral RNA Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturers instructions. cDNA encompassing part of the *pol* gene was produced using Expand<sup>TM</sup> reverse transcriptase (Boehringer Mannheim) as described previously. See Hertogs K., et al., *Antimicrob. Agents Chernother.* 42: 269-276 (1998). A 2.2kb fragment encoding the protease and RT regions was then amplified by nested polymerase chain reaction (PCR) using PCR primers and conditions as described. Id. This genetic material was 25 subsequently used in both phenotyping and genotyping experiments.

25 MT-4 cells (Harada S., et al, *Science* 229: 563-566 (1985).) were co-transfected with *pol* gene PCR fragments and the protease-RT deleted HIV-1 molecular clone, pGEM3\_PRT, as described. See Hertogs K., et al., *Antimicrob. Agents Chernother.* 42: 269-276 (1998). This resulted in viable recombinant viruses containing protease/RT 30 from the donor PCR fragment. Phenotypic susceptibility to nucleoside analogues was determined using an MT-4 cell viral cytopathic effect (CPE) protection assay as described. Id. Fold resistance values were derived by dividing the mean IC<sub>50</sub> for a patient's recombinant virus by the mean IC<sub>50</sub> for wild-type control virus (strain HXB2-D). The PCR products obtained from patient plasma samples were genotyped by 35 dideoxynucleotide-based sequence analysis. Samples were sequenced using the BigDye<sup>TM</sup> terminator kit (Applied Biosystems, Inc. (ABI)) and resolved on an ABI 377 DNA sequencer.

Mutations in the RT coding region were created by site-directed mutagenesis of a wild-type HXB2-D *EcoRI-PstI* restriction enzyme fragment, encompassing the HIV-1 *pol* gene and cloned into pGEM3 (Promega). Single and multiple nucleotide changes were introduced into RT using the ExSite<sup>TM</sup> mutagenesis kit (Stratagene). All mutant 5 clones were verified by DNA sequence analysis of the entire RT. PCR fragments were prepared from the mutated clones and the altered RT coding regions were transferred into the HIV-1 HXB2-D genetic background by homologous recombination as described above. The susceptibility of these recombinant viruses to nucleoside analogues was subsequently determined by the MT-4 cell CPE protection assay. Id.

10 **Phenotypic susceptibility analysis**

The recombinant virus assay (Antivirogram<sup>®</sup>) was used to determine simultaneously the susceptibility of the samples to AZT, 3TC, d4T ddI and ddC. From this analysis, 302 samples were identified with four-fold or greater increases in IC<sub>50</sub> (relative to a wild-type control virus) to at least four of these inhibitors. Thus, a 15 substantial number of MDR viruses were present in the sample population.

**Genotypic analysis of multi-nucleoside resistant samples**

Genotypic analysis was performed on all 892 samples by dideoxy-nucleotide sequencing. Complex patterns of multiple mutations were seen in the RT coding regions of the MDR samples. These included combinations of AZT and 3TC 20 resistance mutations (particularly 41L, 67N, 210W and 215Y with 184V/I) plus mutations at codons 69 (T69A/N) and/or 75 (V75M). This analysis highlighted the incidence of the codon 151 mutational cluster in the MDR group. In addition, a novel family of amino acid insertions and rearrangements between codons 67 and 70 were also prevalent in the MDR group. These two patterns of mutations were correlated with 25 high-level phenotypic multi-nucleoside resistance (Fig. 2), 27 samples having the codon 151 cluster and 45 samples having insertions and rearrangements (typically a T69S substitution, followed by insertion of two amino acids). The mean fold increases in IC<sub>50</sub> to d4T, ddI and ddC for these different groups are shown in Fig. 2. This analysis indicated that codon 69 insertion mutants had a high degree of d4T and ddC resistance 30 (>10-fold), which was also seen with the codon 151 cluster. However, samples with AZT and 3TC resistance mutations plus T69A/N or V175M showed only modest levels of resistance to these drugs (Fig. 2). Not surprisingly, all four groups shown in Fig. 2 were highly resistant to AZT and 3TC (mean fold increase in AZT IC<sub>50</sub> of >500-fold and >30-fold for 3TC). This was because many MDR samples contained 35 mutations conferring AZT resistance (e.g., 41L, 67N, 210W and 215Y) and 3TC resistance (Met184V/I).

**Spectrum of different insertions seen in the RT codon 67 to 70 region**

The extensive variety of insertions in the, codon 67 to 70 region of RT is summarized in Table 3. The largest group (n=16) had a T69S substitution followed by insertion of two S residues. The next largest group (n=10) also had a T69S substitution but in this case a different insertion of S-G. Samples with a number of different double 5 amino acids inserted after 69Ser were also identified. In addition, insertions of two or three amino acids between codons 68 and 69 were also seen. The positions of these insertions were based on the fact that T69 and L70 were contiguous. In some samples there were rarely observed substitutions at codon 67 (A67G/S/G), rather than the common 67N AZT resistance mutation. In two samples deletion of codon 70 was 10 observed (after insertion of three residues between codons 68 and 69), and a single substitution of T69S without an insertion was seen in four samples (Table 3). The inserted residues did not show any obvious patterns in terms of codon usage. For example, the S-S insertions were rarely direct repeats of the S69 codon, suggesting that simple reiterations of S69 could not account for the appearance of these insertions in 15 the RT.

#### **Patients' therapy patterns in relation to codon 69 insertions**

The codon 69 insertions were always present in a background of AZT resistance mutations, especially T215Y/F. This may not be surprising as therapy histories from many of the patients whose samples were analyzed in this study 20 revealed a common pattern of AZT therapy, followed by combination therapy with nucleosides and protease inhibitors (data not shown). Fig. 3 shows typical treatment patterns for three patients, indicating the time samples were obtained for virological analysis. It was not possible from these histories to determine precisely the nucleoside analog(s) responsible for selecting codon 69 insertions. Sequential samples from patient 25 1 revealed an interesting transition of 69S-[S-S] to 69S-[S-G] during a period of 3TC/d4T combination therapy.

#### **Susceptibility analysis of HIV-1 variants constructed by site-directed mutagenesis**

To investigate the significance of the observed mutational patterns correlated 30 with MDR virus we constructed a series of viruses by site-directed mutagenesis with specific changes in a defined genetic background (HXB2-D). T69A or V75M in a background of AZT mutations conferred little or no resistance to 3TC, d4T, ddI or ddC. Variants were also constructed with 69S-[S-S], either alone or together with two AZT 35 resistance mutations (210W and 215Y). In addition, the potential role of A62V, a substitution also frequently correlated with the insertions was investigated by adding this mutation to a background of 69S-[S-S] plus 210W/215Y. Susceptibility data for six nucleoside analogues are summarized in Table 4. These data showed that the 69S-[S-S]

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insertion alone did not confer multi-nucleoside resistance. In fact, this virus only had a significant decrease in susceptibility to 3TC. By contrast, the variants with the insert plus AZT resistance mutations had decreased susceptibility to AZT, 3TC, d4T, ddC and abacavir (4-[(2-amino-6-cyclopropyl-amino)-9H-purin-9-yl]-2-cyclopentene-1-methanol, 1592U89), confirming that the 69 insertions plus AZT mutations conferred the MDR phenotype.

**Example 3: Use of neural networks to define the genetic basis of HIV-1 resistance to d4T.**

Three neural network models (the 9RT, 26RT and 60RT models) were developed to investigate how mutation patterns influence d4T resistance. The 9 RT model was based on the nine mutations listed in the Stanford sequence database (<http://www.hivb.stanford.edu>) associated with d4T resistance (62V, 69D, 69N, 69SXX, 75I, 75T, 77L, 116Y, and 151M). The other models were based on adding either the next 17 or 51 most frequent RT mutations present in d4T resistant samples. Thus, the 26 RT mutation model included the 9 RT mutation model plus the 17 most frequent mutations in d4T resistant samples. These 17 mutations were 41L, 44D, 67N, 70R, 75A, 75M, 115F, 118I, 184V, 208Y, 210W, 214F, 215F, 215Y, 219E, 219N, and 219Q. The 60 RT mutation model consisted of the 26 RT mutation model, plus the 34 next most frequent mutations in d4T resistant samples. These 34 mutations were 20R, 35I, 39A, 43E, 60I, 65R, 122K, 123E, 135T, 162C, 177E, 196E, 200A, 207E, 211K, 228H, 272A, 277K, 286A, 293V, 297K, 329L, 356K, 357T, 358K, 359S, 360T, 371V, 375V, 376A, 386I, 390R, 399D, and 400A. In order to discover which mutations had contributed to this improved prediction, improved sample IS9-26 and IS9-60 were identified by comparing the phenotypic outputs of the 9-model and 26-model, and the 9-model and 60-model on the test set. The corresponding genotypes of the improved samples were collected and analyzed, all extra mutations contained in the improved samples were screened out, and the frequency of each mutation found in IS9-26 and IS9-60 was calculated and compared with that of the mutation being found in the whole samples. All mutations with higher difference of two frequencies were identified and considered to play a role in conferring resistance to d4T. In this example, the threshold frequency was set to 9%. The following mutations were identified from the 9- and 26-models: 41L (44%-79%), 44D (13%-26%), 67N (36%-56%), 70R (21%-30%), 118I (21%-36%), 210W (34%-65%), and 215Y (44%-81%). The following mutations were identified from the 9- and 60-models: 41L (44%-73%), 67N (36%-56%), 181I (21%-32%), 210W (34%-62%), 211K (49%-59%), and 215Y (44%-74%). In conclusion, these results show that at least 17 RT mutations (the 8 identified here plus the 9 identified above from the Stanford Database) may confer d4T resistance, including AZT

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resistance mutations. The results also identified 10 other mutations that may also confer resistance: 184V(36%-42%), 214F(88%-94%), 75A(0.7%-0.6%), 75M(4%-8%), 115F(1%-0.2%), 208Y(13%-21%), 215F(9%-11%), 219E(5%-4%), 219N(4%-11%), and 219Q(12%-16%).

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Example 4

Overview of the mean fold increase in resistance and the effect of the 101 mutations in the HIV reverse transcriptase gene sequence. The fold increase in resistance is calculated from the increase in the mean IC<sub>50</sub> of the drug relative to the mean IC<sub>50</sub> of the same drug 10 for a wild-type reference laboratory HIV-1 strain. The effect of the mutations was investigated on Nevirapine, Delavirdine and Efavirenz. The results are displayed as mean fold increase and the standard deviation thereof.

Genotype	Nevirapine	Delavirdine	Efavirenz
WT	1.3	1.8	0.9
<i>n</i>	20	19	20
<i>Stdev</i>	0.5	0.6	0.8
101E	4.9	7.7	2.4
<i>n</i>	5	5	5
<i>Stdev</i>	5.8	20.4	2.7
101Q	1.9	1.2	0.9
<i>n</i>	5	5	5
<i>Stdev</i>	2.4	1.5	1.0
101P	53.4	162.4	84.2
<i>n</i>	20	17	18
<i>Stdev</i>	14.3	77.8	81.4
101H	24.0	45.5	3.5
<i>n</i>	23	23	23
<i>Stdev</i>	14.8	65.4	3.1

15 Table 5: Overview of the reverse transcriptase mutations at position 101 and their corresponding influence on the resistance towards reverse transcriptase inhibitors. The data are expressed as fold increase in resistance compared to a laboratory reference strain (WT).

20 Example 5

Overview of the mean fold increase in resistance and the effect of the 103 mutations in the HIV reverse transcriptase gene sequence. The fold increase in resistance is calculated

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from the increase in the mean  $IC_{50}$  of the drug relative to the mean  $IC_{50}$  of the same drug for a wild-type reference laboratory HIV-1 strain. The results are displayed as mean fold increase and the standard deviation thereof.

Genotype	Nevirapine	Delavirdine	Efavirenz
WT	<b>1.3</b>	<b>1.8</b>	<b>0.9</b>
<i>n</i>	20	19	20
<i>Stdev</i>	0.5	0.6	0.8
<b>103N</b>	<b>48.1</b>	<b>176.1</b>	<b>486.8</b>
<i>n</i>	10	10	10
<i>Stdev</i>	13.8	83.8	153.3
<b>103R</b>	<b>1.1</b>	<b>1.6</b>	<b>0.6</b>
<i>n</i>	5	5	5
<i>Stdev</i>	2.8	2.1	1.3
<b>103T</b>	<b>43.7</b>	<b>84.5</b>	<b>2.8</b>
<i>n</i>	4	4	4
<i>Stdev</i>	32.0	61.7	1.2
<b>103H</b>	<b>69.4</b>	<b>191.6</b>	<b>52.7</b>
<i>n</i>	5	5	5
<i>Stdev</i>	16.5	105.2	23.4
<b>103S</b>	<b>45.4</b>	<b>18.3</b>	<b>8.4</b>
<i>n</i>	19	15	18
<i>Stdev</i>	9.0	9.6	3.8

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Table 6: Overview of the reverse transcriptase mutation at position 103 and their corresponding influence on the resistance towards reverse transcriptase inhibitors. The data are expressed as mean fold increase in resistance compared to a laboratory reference strain (WT).

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#### Example 6

Overview of the mean fold increase in resistance and the effect of the 181 mutations in the HIV reverse transcriptase gene sequence. The fold increase in resistance is calculated from the increase in the mean  $IC_{50}$  of the drug relative to the mean  $IC_{50}$  of the same drug for a wild-type reference laboratory HIV-1 strain. The results are displayed as mean fold increase and the standard deviation thereof.

Genotype	Nevirapine	Delavirdine	Efavirenz
WT	1.3	1.8	0.9

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Genotype	Nevirapine	Delavirdine	Efavirenz
<i>n</i>	20	19	20
<i>Stdev</i>	0.5	0.6	0.8
<b>181C</b>	<b>56.0</b>	<b>82.5</b>	<b>2.0</b>
<i>n</i>	11	11	11
<i>Stdev</i>	8.4	59.2	0.8
<b>181I</b>	<b>63.8</b>	<b>81.7</b>	<b>2.0</b>
<i>n</i>	5	5	5
<i>Stdev</i>	20.1	57.9	1.0
<b>181S</b>	<b>82.8</b>	<b>147.9</b>	<b>8.7</b>
<i>n</i>	5	5	4
<i>Stdev</i>	21.7	64.4	3.5

Table 7: Overview of the reverse transcriptase mutation at position 181 and their corresponding influence on the resistance towards reverse transcriptase inhibitors. The data are expressed as fold increase in resistance compared to a laboratory reference strain (WT).

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#### Example 7

Overview of the mean fold increase in resistance and the effect of the 190 mutations in the HIV reverse transcriptase gene sequence. The fold increase in resistance is calculated 10 from the increase in the mean  $IC_{50}$  of the drug relative to the mean  $IC_{50}$  of the same drug for a wild-type reference laboratory HIV-1 strain. The results are displayed as mean fold increase and the standard deviation thereof.

Genotype	Nevirapine	Delavirdine	Efavirenz
<b>WT</b>	<b>1.3</b>	<b>1.8</b>	<b>0.9</b>
<i>n</i>	20	19	20
<i>Stdev</i>	0.5	0.6	0.8
<b>190A</b>	<b>60.7</b>	<b>2.3</b>	<b>24.8</b>
<i>n</i>	4	4	4
<i>Stdev</i>	12.6	0.7	11.3
<b>190S</b>	<b>75.9</b>	<b>1.9</b>	<b>483.6</b>
<i>n</i>	6	6	6
<i>Stdev</i>	21.8	3.4	231.1
<b>190E</b>	<b>40.0</b>	<b>131.1</b>	<b>385.3</b>
<i>n</i>	24	22	24
<i>Stdev</i>	7.3	61.2	22.0

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Genotype	Nevirapine	Delavirdine	Efavirenz
190Q	52.7	34.0	401.1
n	22	21	22
Stdev	8.8	58.9	22.5

Table 8: Overview of the reverse transcriptase mutation at position 190 and their corresponding influence on the resistance towards reverse transcriptase inhibitors. The data are expressed as fold increase in resistance compared to a laboratory reference strain (WT).

Example 8. Fold increase in resistance of protease inhibitors by the 84A mutation (in a background)

Table 9: Overview of the mean fold increase in resistance and the effect of the 84A mutation in the HIV protease sequence. The fold increase in resistance is calculated from the increase in the mean IC<sub>50</sub> of the drug relative to the mean IC<sub>50</sub> of the same drug for a wild-type reference laboratory HIV-1 strain.

#	Genotype	Indinavir	Ritonavir	Nelfinavir	Saquinavir	Amprenavir
1	WT	0.9	0.6	0.7	0.9	1
	n	6	6	6	6	6
	Stdev	0.5	0.4	0.2	0.2	0.4
2	10I	0.7	1.15	1	0.9	1.1
	n	4	4	4	4	4
	Stdev	0.2	0.7	0.6	0.3	0.4
3	46I	0.6	0.8	1.5	0.6	1.2
	n	5	5	5	5	5
	Stdev	0.6	0.8	0.9	0.2	0.2
4	46I + 84V	2.6	4.6	5.6	2.5	2.3
	n	2	2	2	2	2
	Stdev	2.1	1.6	4.5	2.4	3.0
5	46I + 84A	26.8	51.8	61.9	38.1	7.7
	n	5	5	5	5	5
	Stdev	20.9	39.1	18.1	11	25.3
6	10I + 46I	2.9	1.5	0.8	0.5	5.6
	n	5	5	5	5	5
	Stdev	3.7	0.3	0.7	0.3	2.9
7	10I + 46I + 84V	2.4	11.9	3.1	4.1	4.5
	n	4	4	4	4	4

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#	Genotype	Indinavir	Ritonavir	Nelfinavir	Saquinavir	Amprenavir
	<i>Stdev</i>	1.5	7.1	3.1	2.7	4.1
8	<b>10I + 46I + 84A</b>	33.4	77.3	43.9	48.2	17
	<i>n</i>	5	5	5	5	5
	<i>Stdev</i>	29.9	38.3	25	9.6	31.1
9	<b>10L/I + 46I + 84A</b>	44.5	114	56.2	46.4	24.9
	<i>n</i>	5	5	5	5	5
	<i>Stdev</i>	22.9	44.4	12.5	9.5	8.2
10	<b>10I + 46I + 84A</b>	26	44.5	36.9	42.8	11
	<i>n</i>	4	5	5	5	5
	<i>Stdev</i>	11	20	24.7	9.7	5
11	<b>46I + 71V</b>	3.7	1.4	1	0.6	2.2
	<i>n</i>	5	5	5	5	4
	<i>Stdev</i>	2.3	0.8	0.1	0.5	1.8
12	<b>46I + 71V + 84V/I</b>	3.1	1.8	0.7	0.6	2.2
	<i>n</i>	1	1	1	1	1
	<i>Stdev</i>					
13	<b>46I + 71V + 84A</b>	56.5	102.2	59.9	47.7	23
	<i>n</i>	6	6	6	6	5
	<i>Stdev</i>	22.8	28	21.3	7.1	12.6
14	<b>10F + 46I + 71V</b>	22.2	4.1	45.2	1.9	4.2
	<i>n</i>	5	5	5	5	4
	<i>Stdev</i>	1.9	2.1	16.1	1.1	2.2
15	<b>10I + 46I + 71V + 84V</b>	50.5	45.6	57.1	15.9	17.8
	<i>n</i>	14	14	14	14	12
	<i>Stdev</i>	26.0	29.8	17.4	8.6	11.3
16	<b>10I + 46I + 71V + 84A</b>	45.9	114.9	54.9	48.2	15.2
	<i>n</i>	5	5	5	5	5
	<i>Stdev</i>	28.3	42.9	21.8	13.1	2.5
17	<b>10V + 46I + 71T + 84A</b>	57.9	91.9	51.2	48.2	39.8
	<i>n</i>	5	5	5	5	5
	<i>Stdev</i>	21.2	66.8	22.2	7.9	24.7
18	<b>10I + 46I + 71V +</b>	2.2	1.65	6.1	1.3	0.9

#	Genotype	Indinavir	Ritonavir	Nelfinavir	Saquinavir	Amprenavir
	<b>77I</b>					
	<b>n</b>	6	6	5	6	6
	<b>Stdev</b>	2.6	0.7	1.8	0.8	0.4
19	<b>10I + 46I + 71V +</b>	2.2	3.1	2.8	2.5	1.4
	<b>77I + 84V</b>					
	<b>n</b>	3	3	3	3	3
	<b>Stdev</b>	5.2	2.1	8.0	2.1	0.5
20	<b>10I + 46I + 71V +</b>	57.9	121.3	68.6	47.1	15
	<b>77I + 84A</b>					
	<b>n</b>	5	5	5	5	4
	<b>Stdev</b>	22.2	71.4	22.1	6.1	6.6
21	<b>10I + 33L/I + 46I</b>	31.4	38.6	63.1	44.7	10.9
	<b>+ 71V + 77V/I</b>					
	<b>+84A</b>					
	<b>n</b>	5	5	6	6	5
	<b>Stdev</b>	16	14.9	13.5	6	5.5

This table exemplifies that 84A mutation in a background of PR mutations confers an additional effect towards the protease inhibitors investigated. This indicates that even in a complex background of mutations the 84A mutation has an effect. The 84A mutation displays a different resistance profile compared with 84V, and teaches that not only the presence but also the exact identity of the mutation is important.

- 5
- Example 9.** Fold increase in resistance of protease inhibitors by the 84L mutation (in a background)
- 10 Table 10 Overview of the mean fold increase in resistance and the effect of the 84L mutation in the HIV-1 protease sequence. The fold increase in resistance is calculated from the increase in the mean IC50 of the drug relative to the mean IC50 of the same drug for a wild-type reference laboratory HIV-1 strain.

#	Genotype	Indinavir	Ritonavir	Nelfinavir	Saquinavir	Amprenavir
1	<b>10I + 36I + 71V</b>	1.4	1.2	1.5	1.6	1.0
	<b>n</b>	6	6	6	6	6
	<b>Stdev</b>	0.5	0.5	0.6	0.8	0.6
2	<b>10I + 36I + 71I + 84V</b>	1.0	1.5	0.8	0.8	1.1
	<b>n</b>	1	1	1	1	1
	<b>Stdev</b>					

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3	10I + 36I + 71I + 84L	3.7	0.8	39.4	10.2	0.3
	<i>n</i>	4	5	5	5	5
	<i>Stdev</i>	3.4	0.9	17.4	6.5	0.2

Genotyping of the sample displays that the 84L mutation is linked to protease resistance as compared to the result obtained from the 84V mutation in the same background.

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**Example 10** Fold increase in resistance of protease inhibitors by the 84C mutation (and in a background)

Table. 11 Overview of the mean fold increase in resistance and the effect of the 84C mutation in the HIV protease sequence. The fold increase in resistance is calculated from the increase in the mean IC50 of the drug relative to the mean IC50 of the same drug for a wild-type reference laboratory HIV-1 strain.

#	Genotype	Indinavir	Ritonavir	Nelfinavir	Saquinavir	Amprenavir
1	WT	0.9	0.6	0.7	0.9	1.0
	<i>n</i>	6	6	6	6	6
	<i>Stdev</i>	0.5	0.4	0.2	0.2	0.4
2	84V	0.7	2.4	0.9	0.7	2.0
	<i>n</i>	9	9	9	9	2
	<i>Stdev</i>	0.9	2.0	0.8	0.6	0.9
3	84C	2.7	2.9	51.2	26.4	5.9
	<i>n</i>	5	5	5	4	5
	<i>Stdev</i>	1.4	3.2	16.2	6.8	3.1
4	10I	0.7	1.2	1.0	0.9	1.1
	<i>n</i>	4	4	4	4	4
	<i>Stdev</i>	0.2	0.7	0.6	0.3	0.4
5	10I + 84V	1.8	6.5	2.0	2.7	1.2
	<i>n</i>	3	3	3	3	3
	<i>Stdev</i>	1.1	2.9	3.1	1.3	0.3
6	10I + 84C	4.3	5.4	61.9	37.1	3.4
	<i>n</i>	7	7	7	7	5
	<i>Stdev</i>	3.2	3.8	18.0	16.5	1.5
7	77I	0.4	0.4	0.9	0.3	0.6
	<i>n</i>	3	3	3	3	3
	<i>Stdev</i>	0.1	0.3	0.3	0.1	0.3

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#	Genotype	Indinavir	Ritonavir	Nelfinavir	Saquinavir	Amprenavir
8	77I + 84V	2.5	5.0	2.8	3.5	3.3
	<i>n</i>	1	1	1	1	1
	<i>Stdev</i>					
9	77I + 84C	3.1	2.8	61.9	18.1	2.2
	<i>n</i>	5	4	5	5	5
	<i>Stdev</i>	1.7	1.3	24.1	13.3	1.2
10	77I + 84C	3.2	3.2	38.2	18.5	4.9
	<i>n</i>	5	6	6	5	6
	<i>Stdev</i>	2.9	1.4	22.3	12.1	2.5
11	10I + 24I + 36I + 46I + 71V + 84C	7.5	47	51.7	46.4	12.8

The unique 84C mutation confers resistance towards protease inhibitors. The appearance of the 84C mutation is indicative of but not limited to Nelfinavir resistance.

5   **Example 11**   Fold increase in resistance of protease inhibitors by the 54M mutation  
(in a background)

Table 12: Influence of the 54M mutation in a background of different protease mutations and their corresponding effect on protease inhibitor resistance. The effect for the investigated compounds is expressed as a mean fold change in IC<sub>50</sub> towards the mean IC<sub>50</sub> determined for a wild type laboratory HIV strain.

Virco ID	Genotype	Indinavir Fold change in IC <sub>50</sub>	Ritonavir Fold change in IC <sub>50</sub>	Nelfinavir Fold change in IC <sub>50</sub>	Saquinavir Fold change in IC <sub>50</sub>	Amprenavir Fold change in IC <sub>50</sub>
V021667	10I	0.7	1.2	1.0	0.9	1.1
V053832	10I+33F	0.4	2.9	1.6	0.5	0.6
V052977	10I+33F+54M	1.8	35.5	3.8	0.5	12.2
V048878	10I+33F+54M+71V +77I+90M	5.2	17.2	7.3	4.0	13.7
V048879	10I+33F+54M+71V +77I+90M	6.5	74.0	45.7	51.8	14.1
V052978	10I+33F+54M+71V +90M	5.4	17.9	29.1	9.9	19.3
V055199	10I+33F+54M+90M	2.3	18.4	11.7	5.6	38.7
V052953	10I+33F+77I	0.8	1.1	0.9	1.2	1.5

Virco ID	Genotype	Indinavir Fold change in IC <sub>50</sub>	Ritonavir Fold change in IC <sub>50</sub>	Nelfinavir Fold change in IC <sub>50</sub>	Saquinavir Fold change in IC <sub>50</sub>	Amprenavir Fold change in IC <sub>50</sub>
V048880	10I+33F+77I+90M	1.3	3.6	4.9	2.7	3.8
V048877	10I+33F+77I+90M	2.9	20.5	9.4	1.5	7.9
V052979	10I+54M	1.8	20.0	3.8	1.4	10.4
V052982	10I+54M+71V+77I+ 90M	2.4	13.4	10.1	4.2	3.1
V052981	10I+54M+71V+90M	3.2	9.4	8.7	5.4	8.9
V048869	10I+54M+77I+90M	7.1	20.0	28.5	5.1	9.3
V052980	10I+54M+90M	1.3	4.1	7.0	1.4	1.4
V052942	10I+71V	0.6	0.6	0.9	0.8	0.5
V052949	10I+71V+77I+90M	2.7	2.3	8.6	2.1	0.6
V052948	10I+71V+90M	8.6	10.5	9.0	4.8	2.0
V030496	10I+77I	0.4	0.5	0.7	0.3	0.5
V052947	10I+77I+90M	2.9	6.9	8.2	3.9	1.2
V052943	10I+90M	0.5	1.8	2.2	1.1	0.4
V052944	10I+90M	0.5	1.5	1.4	0.7	0.7
V052950	33F	0.6	1.5	1.0	1.2	0.7
V048872	33F+54M+77I+90M	3.2	8.7	7.4	5.2	30.3
V048873	33F+54M+77I+90M	1.7	11.4	7.7	3.1	37.5
V052955	33F+71V+90M	5.3	77.9	36.6	20.4	2.7
V052951	33F+77I	0.4	0.3	5.2	0.4	0.3
V052954	33F+77I+90M	1.7	2.2	5.9	1.7	2.1
V052940	71V	0.7	0.4	0.6	0.8	0.3
V052946	71V+90M	0.9	2.0	3.0	1.4	0.5
V19263	77I	0.4	0.4	0.9	0.3	0.6
V052945	77I+90M	0.6	1.9	3.0	1.4	0.3
V20160	WT	0.9	0.6	0.7	0.9	1.0

**Example 12** Fold increase in resistance of protease inhibitors by the 54L mutation (in a background)

5 Table 13: Influence of 54L mutation in a background of different protease mutations and their corresponding effect on protease inhibitor resistance. The effect for the investigated compounds is expressed as a mean fold change in IC<sub>50</sub> towards the mean IC<sub>50</sub> determined for a wild type laboratory HIV strain.

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Virco ID	Genotype	Indinavir Fold change in IC <sub>50</sub>	Ritonavir Fold change in IC <sub>50</sub>	Nelfinavir Fold change in IC <sub>50</sub>	Saquinavir Fold change in IC <sub>50</sub>	Amprenavir Fold change in IC <sub>50</sub>
V021667	10I	0.7	1.2	1.0	0.9	1.1
V053832	10I+33F	0.4	2.9	1.6	0.5	0.6
V048875	10I+33F+54L+71V+ 77I+90M	3.7	25.1	12.5	12.0	24.6
V048874	10I+33F+54L+71V+ 77I+90M	2.1	21.3	14.5	4.6	32.1
V052969	10I+33F+54L+90M	1.3	4.8	3.4	1.4	10.4
V052953	10I+33F+77I	0.8	1.1	0.9	1.2	1.5
V048880	10I+33F+77I+90M	1.3	3.6	4.9	2.7	3.8
V048877	10I+33F+77I+90M	2.9	20.5	9.4	1.5	7.9
V052971	10I+54L	0.7	1.5	1.0	0.7	2.6
V052976	10I+54L+71V+77I+9 0M	1.7	6.5	8.9	1.8	2.0
V048868	10I+54L+71V+77I+9 0M	6.2	9.7	15.6	16.5	4.1
V052975	10I+54L+71V+90M	10.2	30.5	41.3	24.2	6.2
V052973	10I+54L+77I	0.8	1.4	2.8	0.8	2.1
V052974	10I+54L+90M	1.7	3.6	2.6	1.1	1.2
V052942	10I+71V	0.6	0.6	0.9	0.8	0.5
V052949	10I+71V+77I+90M	2.7	2.3	8.6	2.1	0.6
V052948	10I+71V+90M	8.6	10.5	9.0	4.8	2.0
V030496	10I+77I	0.4	0.5	0.7	0.3	0.5
V052947	10I+77I+90M	2.9	6.9	8.2	3.9	1.2
V052943	10I+90M	0.5	1.8	2.2	1.1	0.4
V052944	10I+90M	0.5	1.5	1.4	0.7	0.7
V052968	10L/I+33F/L+54L+ 77I	1.3	4.7	3.5	1.5	2.9
V052950	33F	0.6	1.5	1.0	1.2	0.7
V052955	33F+71V+90M	5.3	77.9	36.6	20.4	2.7
V052951	33F+77I	0.4	0.3	5.2	0.4	0.3
V052954	33F+77I+90M	1.7	2.2	5.9	1.7	2.1
V052970	54L	0.6	2.0	1.6	0.7	0.8
V053854	54L+71V	1.7	2.3	5.9	0.9	5.0
V052972	54L+77I	0.8	2.4	3.0	1.0	1.1

Virco ID	Genotype	Indinavir Fold change in IC <sub>50</sub>	Ritonavir Fold change in IC <sub>50</sub>	Nelfinavir Fold change in IC <sub>50</sub>	Saquinavir Fold change in IC <sub>50</sub>	Amprenavir Fold change in IC <sub>50</sub>
V052940	71V	0.7	0.4	0.6	0.8	0.3
V052946	71V+90M	0.9	2.0	3.0	1.4	0.5
V19263	77I	0.4	0.4	0.9	0.3	0.6
V052945	77I+90M	0.6	1.9	3.0	1.4	0.3
V20160	WT	0.9	0.6	0.7	0.9	1.0

**Example 13.** Fold increase in resistance of protease inhibitors by the 54V mutation (in a background)

Table 14: Influence of 54V on protease resistance in a background of different mutations  
5 and their corresponding effect on drug resistance. The effect for the investigated compounds is expressed as a mean fold change in IC<sub>50</sub> towards the mean IC<sub>50</sub> determined for a wild type laboratory HIV strain.

Virco ID	Genotype	Indinavir Fold change in IC <sub>50</sub>	Ritonavir Fold change in IC <sub>50</sub>	Nelfinavir Fold change in IC <sub>50</sub>	Saquinavir Fold change in IC <sub>50</sub>	Amprenavir Fold change in IC <sub>50</sub>
V021667	10I	0.7	1.2	1.0	0.9	1.1
V053832	10I+33F	0.4	2.9	1.6	0.5	0.6
214846	10I+33F+54V+71V+ 77I+90M	13.8	57.6	43.5	27.8	4.2
V052960	10I+33F+54V+71V+ 90M	9.9	70.9	40.7	21.8	6.4
V052959	10I+33F+54V+77I+9 0M	12.8	28.5	7.5	1.0	7.0
V052958	10I+33F+54V+77I+9 0M	16.3	16.6	4.8	1.0	7.8
V052957	10I+33F+54V+90M	1.4	13.9	6.9	1.6	5.2
V052953	10I+33F+77I	0.8	1.1	0.9	1.2	1.5
V048880	10I+33F+77I+90M	1.3	3.6	4.9	2.7	3.8
V048877	10I+33F+77I+90M	2.9	20.5	9.4	1.5	7.9
V052962	10I+54V	0.8	1.0	0.6	0.9	0.5
V052967	10I+54V+71V+77I+ 90M	0.4	0.7	0.8	0.6	0.4
V052966	10I+54V+71V+90M	7.7	44.5	55.4	28.6	1.5

Virco ID	Genotype	Indinavir Fold change in IC <sub>50</sub>	Ritonavir Fold change in IC <sub>50</sub>	Nelfinavir Fold change in IC <sub>50</sub>	Saquinavir Fold change in IC <sub>50</sub>	Amprenavir Fold change in IC <sub>50</sub>
V052965	10I+54V+77I+90M	8.2	12.5	15.1	6.3	0.7
V052942	10I+71V	0.6	0.6	0.9	0.8	0.5
V052949	10I+71V+77I+90M	2.7	2.3	8.6	2.1	0.6
V052948	10I+71V+90M	8.6	10.5	9.0	4.8	2.0
V030496	10I+77I	0.4	0.5	0.7	0.3	0.5
V052947	10I+77I+90M	2.9	6.9	8.2	3.9	1.2
V052943	10I+90M	0.5	1.8	2.2	1.1	0.4
V052944	10I+90M	0.5	1.5	1.4	0.7	0.7
V052950	33F	0.6	1.5	1.0	1.2	0.7
V053831	33F+54V	5.4	129.7	5.9	1.0	7.5
V053358	33F+54V+77I+90M	1.2	4.9	2.1	1.3	1.0
V048876	33F+54V+77I+90M	0.6	5.6	1.6	1.5	1.1
V052955	33F+71V+90M	5.3	77.9	36.6	20.4	2.7
V052951	33F+77I	0.4	0.3	5.2	0.4	0.3
V052954	33F+77I+90M	1.7	2.2	5.9	1.7	2.1
V052961	54V	0.7	1.2	1.0	1.0	0.5
V052964	54V+71V+90M	0.7	7.1	3.3	1.4	0.3
V052963	54V+77I+90M	1.2	2.0	3.2	1.3	0.5
V052940	71V	0.7	0.4	0.6	0.8	0.3
V052946	71V+90M	0.9	2.0	3.0	1.4	0.5
V19263	77I	0.4	0.4	0.9	0.3	0.6
V052945	77I+90M	0.6	1.9	3.0	1.4	0.3
V20160	WT	0.9	0.6	0.7	0.9	1.0

All references, patents, and patent applications cited herein are incorporated by reference in their entirety.

It will be apparent to those skilled in the art that various modifications and variations can be made in the compositions and methods of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present description cover the modifications and variations of this invention provided that they come within the scope of the appended claims and their equivalents.

Claims:

1. A computer system comprising at least one database chosen from :
  - (i) a database correlating the presence of at least one mutation in a human immunodeficiency virus (HIV) reverse transcriptase and resistance of at least one strain of HIV to a reverse transcriptase inhibitor, comprising at least one set of records chosen from :
    - a set of records corresponding to a correlation between at least one mutation chosen from 44D, 77L, 115F, 118I, 184V, 208Y, 210W, 211K, 214F, 215F, 215Y, 219E, 219N, and 219Q, and resistance to d4T;
    - a record corresponding to a correlation between mutation 184I and resistance towards lamivudine;
    - a set of records corresponding to a correlation between at least one mutation chosen from 115F and 184V and resistance towards abacavir;
    - a record corresponding to a combination of 62V, 75T, 77L, 116Y and 151M and resistance towards all nucleoside analogues;
    - a set of records corresponding to a correlation between at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, and 190Q and resistance towards nevirapine;
    - a set of records corresponding to a correlation between at least one mutation chosen from 101H, 101P, 103H, 103N, 103S, 103T, 106M, 181C, 181S and 190Q and resistance towards delavirdine;
    - a set of records corresponding to a correlation between at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L and resistance towards efavirenz;
    - a record corresponding to a combination of 184V and 41L and 215Y, wherein the 184V resistance mutation reverses the effect of 41L and 215Y mutations on zidovudine;
    - a record corresponding to a 236L mutation, which increases the sensitivity towards nevirapine;
  - (ii) a database correlating the presence of at least one mutation in a human immunodeficiency virus (HIV) protease and resistance of at least one strain of HIV to a protease inhibitor, comprising a set of records corresponding to a correlation between at least mutation selected from 54L, 54M, 54V and any mutation at codon 84 and resistance towards a protease inhibitor.
- 35 2. A computer system according to claim 1 wherein the mutation at codon 84 is selected from 84A, 84C and 84L.

3. A computer system according to any one of claims 1 to 2 wherein the protease inhibitor is selected from amprenavir, saquinavir, nelfinavir, ritonavir and indinavir.
- 5 4. A computer system according to any one of claims 1 to 3 wherein the at least one mutation in the HIV protease is combined with at least one mutation in the HIV protease at codon 10 and/or codon 90.
- 10 5. A computer system according to any one of claims 1 to 4 wherein the at least one mutation in the HIV protease is combined with at least one mutation in the HIV protease selected from 10I, 20R, 20T, 24I, 33F, 33I, 33L, 36I, 46L, 71T, 71V, 77I, 77V, 82I, 82V or 90M.
- 15 6. A method of evaluating the effectiveness of d4T as an antiviral therapy of an HIV-infected patient comprising:
  - (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 44D, 77L, 115F, 118I, 184V, 208Y, 210W, 211K, 214F, 215F, 215Y, 219E, 219N, and 219Q;
  - 20 (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
7. A method of evaluating the effectiveness of lamivudine as an antiviral therapy of an HIV-infected patient comprising:
  - (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least the mutation 184I;
  - (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
- 30 8. A method of evaluating the effectiveness of abacavir as an antiviral therapy of an HIV-infected patient comprising:
  - (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 115F and 184V;
  - 35 (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.

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9. A method of evaluating the effectiveness of a nucleoside analogue as an antiviral therapy of an HIV-infected patient comprising:
  - (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 62V, 75T, 77L, 116Y and 151M;
  - (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
10. A method of evaluating the effectiveness of nevirapine as an antiviral therapy of an HIV-infected patient comprising:
  - (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L;
  - (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
11. A method of evaluating the effectiveness of delavirdine as an antiviral therapy of an HIV-infected patient comprising:
  - (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 101H, 101P, 103H, 103N, 103S, 103T, 106M, 181C, 181S, 190Q and 236L;
  - (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
12. A method of evaluating the effectiveness of efavirenz as an antiviral therapy of an HIV-infected patient comprising:
  - (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L;
  - (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
13. A method of evaluating the effectiveness of zidovudine as an antiviral therapy of an HIV-infected patient comprising:

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- (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 184V, 41L and 215Y;
  - (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
- 5
14. A method of evaluating the effectiveness of a protease inhibitor as an antiviral therapy of an HIV-infected patient comprising:
- (a) collecting a sample from an HIV-infected patient;
  - (b) determining whether the sample comprises a nucleic acid encoding a HIV protease having at least one mutation chosen from 54L, 54M, 54V and any mutation at codon 84;
  - (c) using the presence of said at least one mutation of step b) to evaluate the effectiveness of said antiviral therapy.
- 10
15. A method according to claim 14 wherein the mutation at codon 84 is selected from 84A, 84C and 84L.
16. A method according to claim 14 or 15 wherein the at least one mutation in the HIV protease is combined with at least one mutation in the HIV protease at codon 10 and/or codon 90.
- 20
17. A method according to any one of claims 14 to 16 wherein the at least one mutation in the HIV protease is combined with at least one mutation in the HIV protease selected from 10I, 20R, 20T, 24I, 33F, 33I, 33L, 36I, 46L, 71T, 71V, 77I, 77V, 82I, 82V or 90M.
- 25
18. A method of identifying a drug effective against drug resistant strains of HIV, comprising :
- i) providing a HIV protease containing at least one mutation chosen from 54L, 54M, 54V or any mutation at codon 84;
  - ii) determining a phenotypic response of said drug to said HIV protease; and
  - iii) using said phenotypic response to determine the effectiveness of said drug.
- 30
- 35 19. A drug identified using the method as claimed in claim 18.
20. The method of claim 18 wherein said phenotypic response is determined using a recombinant virus assay.

21. A method of designing a therapy with d4T for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- 5 (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 44D, 77L, 115F, 118I, 184V, 208Y, 210W, 211K, 214F, 215F, 215Y, 219E, 219N, and 219Q;
- (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
- 10 22. A method of designing a therapy with lamivudine for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least the mutation 184I;
- 15 (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
23. A method of designing a therapy with abacavir for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 115F and 184V;
- 20 (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
24. A method of designing a therapy with a nucleoside analogue for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- 30 (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 62V, 75T, 77L, 116Y and 151M;
- (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
- 35 25. A method of designing a therapy with nevirapine for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;

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- (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L;
- (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
- 5

26. A method of designing a therapy with delavirdine for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- 10 (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 101H, 101P, 103H, 103N, 103S, 103T, 106M, 181C, 181S, 190Q and 236L;
- (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
- 15
27. A method of designing a therapy with efavirenz for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse
- 20 transcriptase having at least one mutation chosen from 101H, 101P, 103H, 103S, 103T, 106M, 181S, 190Q and 236L;
- (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
- 25
28. A method of designing a therapy with zidovudine for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- (b) determining whether the sample comprises a nucleic acid encoding a HIV reverse transcriptase having at least one mutation chosen from 184V, 41L and 215Y;
- 30 (c) using the presence of said at least one mutation of step b) to design the antiviral therapy.
29. A method of designing a therapy with a protease inhibitor for treating a patient infected with HIV comprising:
- (a) collecting a sample from an HIV-infected patient;
- (b) determining whether the sample comprises a nucleic acid encoding a HIV protease having at least one mutation chosen from 54L, 54M, 54V and any mutation at codon 84;
- 35

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(c) using the presence of said at least one mutation of step b) to design the antiviral therapy.

30. A method according to claim 29 wherein the mutation at codon 84 is selected from  
5 84A, 84C and 84L.

31. A method according to claim 29 or 30 wherein the at least one mutation in the HIV protease is combined with at least one mutation in the HIV protease at codon 10 and/or codon 90.

10

32. A method according to any one of claims 29 to 31 wherein the at least one mutation in the HIV protease is combined with at least one mutation in the HIV protease selected from 10I, 20R, 20T, 24I, 33F, 33I, 33L, 36I, 46L, 71T, 71V, 77I, 77V, 82I, 82V or 90M.

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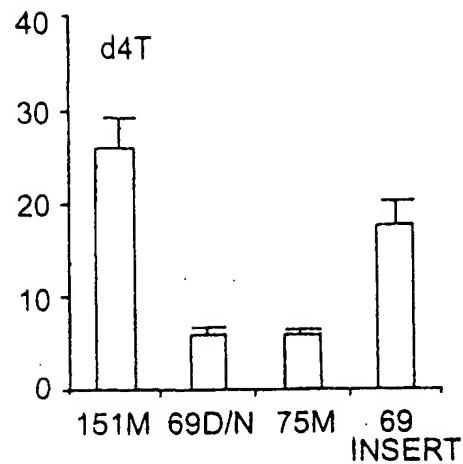


Figure 1a

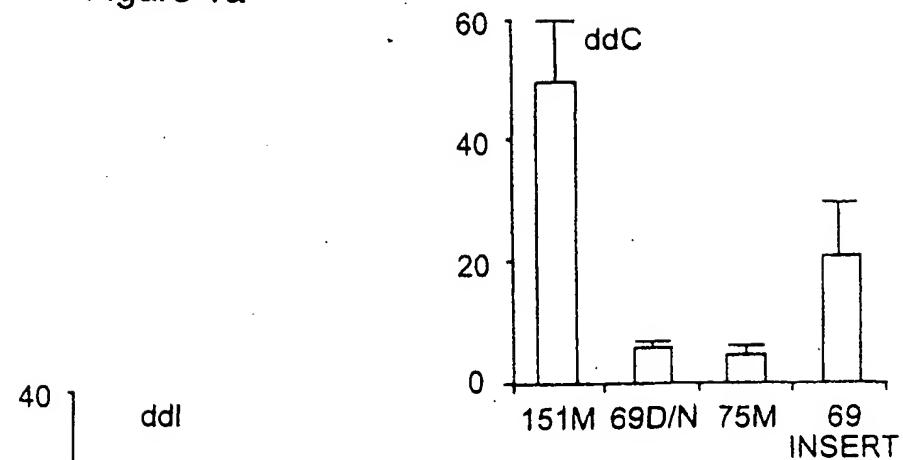


Figure 1b

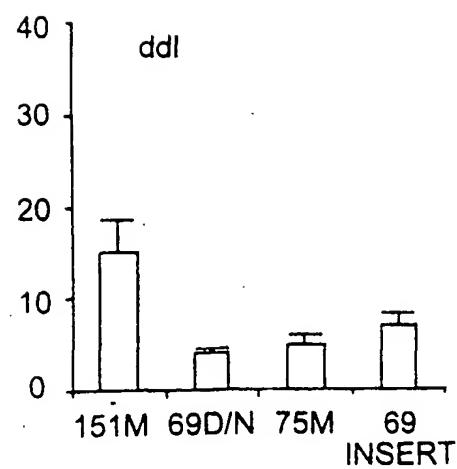


Figure 1c

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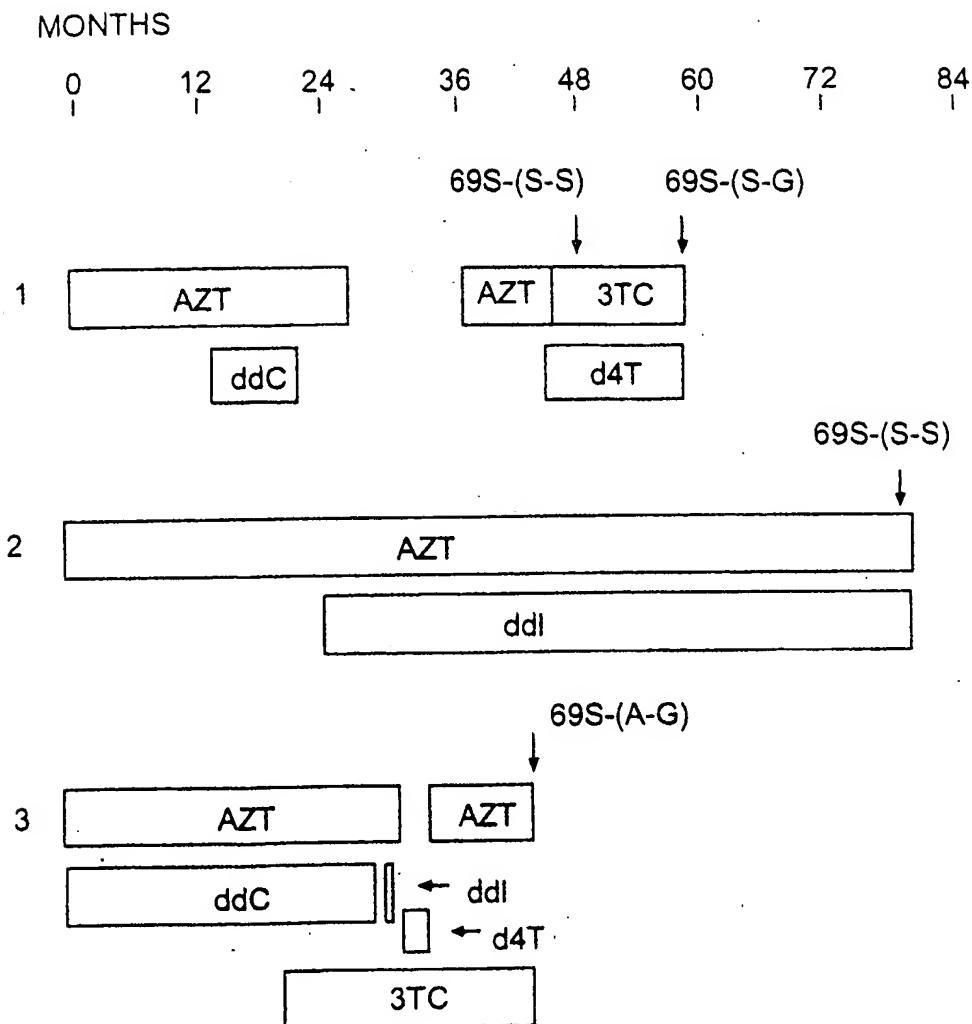


Figure 2